

## C3 PROACTIVATOR CONVERTASE AND ITS MODE OF ACTION\*

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The C3 proactivator (C3PA) is cleaved and converted to an active enzyme capable of acting on C3 when serum is treated with yeast cell walls, inulin, endotoxin, or aggregates of certain immunoglobulins (1). Activation of C3PA was postulated to be effected by a serum enzyme called C3PA convertase (C3Pase). We now wish to report (a) the identification of C3Pase in human serum as a 3S  $\alpha$ -globulin and (b) the finding that C3Pase action requires the presence of a product of C3. The possibility is raised therefore that the C3 activator system is governed by a positive feedback mechanism.

### Materials and Methods

*Isolated Proteins.*—C3 (2) and C3PA (1) were isolated from human serum as described. C3 was utilized at a concentration of 2.6 mg/ml and C3PA at 400  $\mu$ g/ml.

*Isolation of Activated Hydrazine-Sensitive Factor (HSFa).*—The euglobulins of 2.4 liters of outdated plasma were chromatographed on a 5.5  $\times$  67 cm column of DE32 equilibrated with 0.005 M phosphate buffer, pH 7.3. This buffer and all other buffers used contained 0.002 M ethylenediaminetetraacetate (EDTA). HSFa was eluted by NaCl gradient in a conductance range of 7–11 mmho. The active material was then applied to a 2.5  $\times$  95 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with 0.15 M NaCl containing 0.005 M phosphate buffer, pH 7.3. HSFa was eluted with the second protein peak which was further chromatographed using a 2.2  $\times$  34 cm column of BioRex 70 (Bio-Rad Labs., Richmond, Calif.) equilibrated with 0.03 M phosphate buffer, pH 7.0. HSFa was eluted with starting buffer and emerged immediately after the first protein peak.

HSFa was utilized at a concentration of 0.08 mg/ml. 0.2 ml portions were subjected to alkaline disc electrophoresis on 6% gels. The gels were sliced longitudinally; one-half was stained for protein, the other cut into 2 mm segments which were eluted with 0.1 ml Veronal buffer for activity testing. Antiserum to HSFa was made by immunizing rabbits with the isolated protein.

*Purification of C3Pase.*—2  $\times$  10 ml fresh human serum was separated by electrophoresis on Pevikon blocks in barbital buffer, pH 8.6,  $\Gamma/2 = 0.05$ , for 24 hr at 4°C and 3.5 v/cm. Active fractions were pooled and concentrated by ultrafiltration to 2 ml, and the material was further separated by molecular sieve chromatography on a 2.5  $\times$  40 cm column of Sephadex G-150 equilibrated with 0.15 M NaCl in 0.005 M phosphate buffer, pH 7.3. The active fractions were concentrated and portions of 0.2 ml containing 0.085 mg of protein were

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subjected to polyacrylamide gel electrophoresis. The gels were sliced longitudinally; one-half was stained for protein, the other half cut into 2 mm segments which were eluted with 0.1 ml Veronal buffer, pH 7.3, overnight at 4°C. Eluates were analyzed for C3PAse activity and by Ouchterlony test with antisera to the following proteins: Gc (gift of Dr. A. G. Bearn, Cornell University Medical College, New York),  $\alpha_2$ HS glycoprotein,  $\alpha_1$  acid glycoprotein (gifts from Behringwerke AG, Marburg-Lahn, West Germany), and C1s.

*Assay of Hydrazine-Sensitive Factor (HSF).*— $N_2H_4$ -treated human serum was used as a reagent (0.015 M  $N_2H_4$  at 37°C for 60 min). 0.1 ml of serum fraction was added to 0.1 ml of  $N_2H_4$  serum and 0.02 ml 50 mg/ml inulin suspension in sterile saline. The mixture was incubated at 37°C for 30 min and the conversion of C3PA to C3 activator was evaluated by immunoelectrophoresis (IEP) (1). The degree of conversion was estimated visually or by determining the distribution of radioactivity in the IEP slide when C3PA- $^{125}I$  had been added to the serum reagent. Alternatively, HSF activity was determined by lysis of glutathione-treated erythrocytes in presence of  $N_2H_4$  serum and inulin (3).

*Assay of HSFa.*—10  $\mu$ l of HSFa was added to (a) 50  $\mu$ l of whole human serum and 1  $\mu$ l of  $Me^{++}$  (0.015 M  $CaCl_2$  and 0.03 M  $MgCl_2$  in saline), or (b) to 10  $\mu$ l of C3PA, 10  $\mu$ l of C3PAse, and 3  $\mu$ l of  $Me^{++}$ ; the reaction mixtures were held at 37°C for 30 min and evaluated as described above (HSF).

*Assay of C3PAse.*—Test solution (10  $\mu$ l) was added to 10  $\mu$ l of C3PA, 10  $\mu$ l of HSFa, and 3  $\mu$ l of  $Me^{++}$ . Treatment and evaluation were as described above.

*Assay of C3 Conversion by C3 Activator.*—10  $\mu$ l of C3PA, 10  $\mu$ l of C3PAse, 10  $\mu$ l of HSFa, and 5  $\mu$ l of  $Me^{++}$  were incubated with 20  $\mu$ l of C3 for 30 min at 37°C. IEP was performed using monospecific anti-C3.

#### RESULTS

*Identification of HSF.*—Treatment of serum with  $N_2H_4$  prevented conversion of C3PA upon subsequent incubation with inulin. Fractions of untreated serum were therefore tested for their capacity to reconstitute  $N_2H_4$ -treated serum in the inulin test. The distribution of HSF on chromatography of serum using diethylaminoethyl cellulose or Sephadex G-200 was found to correlate with that of C3. When five different preparations of isolated human C3 were tested for HSF activity, they were all found to be active. Further, the distribution of isolated C3 protein upon Pevikon block electrophoresis correlated well with that of HSF activity. It was concluded that HSF in the C3 activator system is identical with native C3, a protein known to be  $N_2H_4$  sensitive (4).

*Isolation of HSFa.*—Exploration of serum fractions for HSF activity led to the recognition of another factor, HSFa, which caused conversion of C3PA in  $N_2H_4$ -treated or untreated serum in absence of inulin. This factor was isolated as described in the Materials and Methods section. On polyacrylamide gel electrophoresis the preparation consisted essentially of one major protein band and the biological activity was eluted exactly from the location of the protein disc (Fig. 1). Tested by the double diffusion in gel method it gave one precipitin line with an anti-whole human serum which fused with the line produced with an anti-C3 and with that of an anti-HSFa. On immunoelectrophoresis HSFa was located more anodal than native C3 (Fig. 1), and in mixture with native C3, both lines fused, with native C3 extending a spur over the HSFa line (not shown). Anti-HSFa when tested with whole human serum reacted only with C3. By sucrose density gradient ultracentrifugation,

the *s* rate of HSFa was estimated to be 8.5S (Fig. 2). Its activity was not abolished by  $N_2H_4$  treatment. Thus the activity to effect conversion of C3PA in whole human serum appears to reside in a fragment of C3 which with respect to *s* rate and electrophoretic behavior resembles C3b. It may be considered the activated form of HSF. In contradistinction to its effect in serum, isolated HSFa was unable to cause conversion of isolated C3PA (Fig. 3).

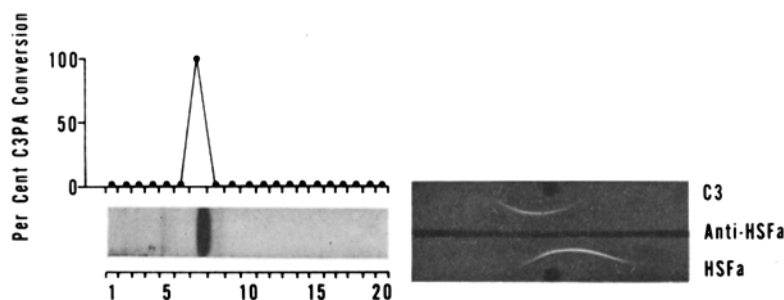


FIG. 1. Analysis of isolated HSFa. (Left): disc electrophoresis on 6% polyacrylamide gel. Anode was at the right. HSFa activity was eluted from segments of the unstained half of gel. (Right): immunoelectrophoresis of isolated C3 (above) and HSFa (below); pattern developed with anti-HSFa. Anode was at the right.

*C3 Proactivator Convertase.*—Using C3PA and HSFa it was now possible to screen serum fractions for C3PAse activity. This activity was found in the  $\alpha$ -globulin region of serum separated by Pevikon block electrophoresis (Fig. 4). On density gradient ultracentrifugation of the  $\alpha$ -globulin fraction or of whole human serum, C3PAse sedimented as a 3S component (Fig. 2). Disc electrophoresis of a partially purified preparation of C3PAse allowed identification of the enzyme with a discrete protein band. Identification with one of the known  $\alpha$ -globulins using various antisera (see Materials and Methods section) has failed so far. C3PAse is distinct from C1s, a 4S  $\alpha$ -globulin, by activity and immunochemical properties.

C3PAse, in partially purified form or eluted from polyacrylamide gels, failed to convert isolated C3PA, but it effected complete conversion of C3PA in the presence of HSFa and metal ions (Fig. 3). 0.01 M EDTA inhibited the reaction, as did heparin (1000 units/ml), and epsilon amino caproic acid (0.5 M) accelerated it. Native C3, when free of conversion products, could not substitute for HSFa. Further, C3PA conversion could not be initiated in mixtures with C3PAse, native C3, and  $Me^{++}$ , by the addition of inulin, which leads to C3PA conversion in whole serum. Native C3-converting activity was generated during incubation at 37°C of C3PA, C3PAse, HSFa, and  $Me^{++}$  (Fig. 3).

#### DISCUSSION

The results reported in this paper have extended our concept of the C3 activator system (Fig. 5). Conversion of C3PA to C3 activator, an enzyme which

## C3 PROACTIVATOR CONVERTASE

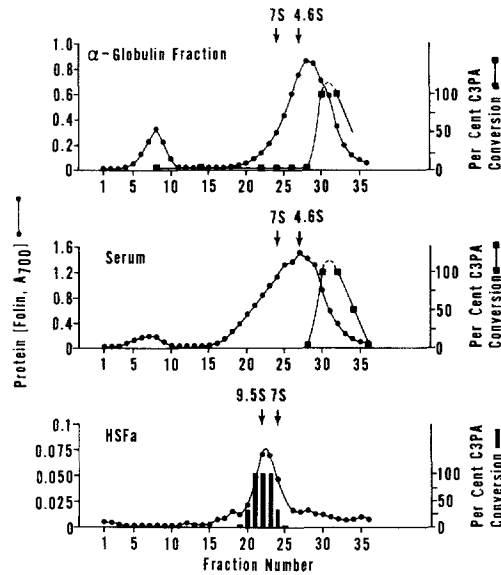


FIG. 2. Sedimentation behavior of C3Pase in  $\alpha$ -globulin fraction (*top*), C3Pase in whole human serum (*middle*), and isolated HSFa (*bottom*). Ultracentrifugation was performed in a linear 7–31% sucrose density gradient at 45,000 rpm in a SW50 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) for 16 hr at 4°C.

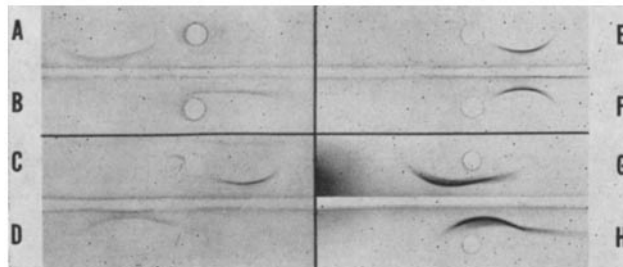


FIG. 3. The mode of action of C3Pase explored by immunelectrophoresis. (A) Treatment of serum with HSFa led to conversion of C3PA; (B) C3PA in serum incubated with buffer was unconverted; (C) partially purified C3Pase and  $\text{Me}^{++}$  failed to convert isolated C3PA; (D) isolated C3PA was converted, however, by C3Pase,  $\text{Me}^{++}$ , and HSFa; (E) this reaction was totally inhibited by 0.01 M EDTA; (F) HSFa and  $\text{Me}^{++}$  were without effect on isolated C3PA; (G) native C3 was unaffected when treated with C3PA, HSFa, and  $\text{Me}^{++}$ ; (H) but totally converted by C3PA, C3Pase, HSFa, and  $\text{Me}^{++}$ . Before application all samples received EDTA (up to 0.02 M) and the agar gel contained 0.01 M EDTA. Patterns A–F were developed with anti-C3PA, G and H with anti-C3. Anode was at the right.

cleaves C3 into C3a and C3b, is accompanied by fragmentation of the C3PA molecule. This reaction is catalyzed by the enzyme C3Pase, a 3S  $\alpha$ -globulin, which for its action on C3PA requires metal ions and a fragment of C3 (HSFa) which might be identical with C3b. The fragment appears to fulfill the function of an enzyme effector or modifier; its precursor, native C3, representing

the previously recognized  $N_2H_4$ -sensitive factor (1). C3b, or a fragment closely resembling it, is thus envisioned to play a key role in the C3 activator system; as one of the final products of the reaction it can "switch on" C3PAse and thereby initiate a positive feedback mechanism.

An effector function of a protein fragment in the complement system is not without precedent. Thus, C4i has been shown to uncover the specificity of

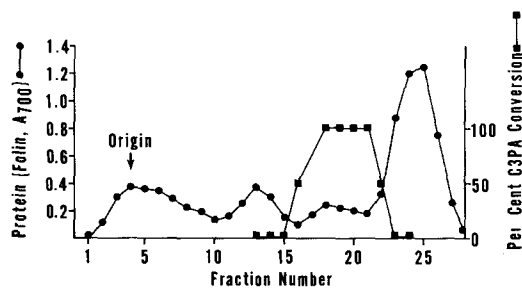


FIG. 4. Detection of C3PAse in electrophoretically separated human serum. Conditions of electrophoresis: Pevikon block in barbital buffer, pH 8.6, ionic strength 0.05, containing 0.002 M EDTA, 3.5 v/cm for 23 hr at 4°C.

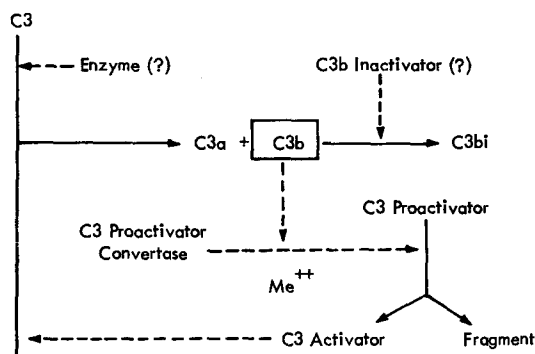


FIG. 5. Hypothetical concept of a portion of the C3 activator mechanism proposing that a C3 fragment (here designated C3b) has an effector function for C3PAse. The possible control of this fragment by a serum factor is indicated (C3b inactivator?). The initiation of the reaction by inulin, etc., requires additional serum factor(s) (enzyme?).

C1 for C2 (5), and a peptide related to kallikrein was found to enhance C4,2-forming activity of C1 (6). Most pertinent to the present study is the effect of C3b on C4,2; in modifying this enzyme C3b enables it to act on C5 (4).

How substances like inulin set in motion the proposed mechanism when added to serum is still unknown. A direct action of inulin on C3PAse was ruled out by the reported experiments. Perhaps inulin acts together with an as yet unidentified serum factor (enzyme?) and C3 on C3PAse. That the missing factor might be properdin has been considered (1), especially since the activity of our isolated C3PA could be equated with Factor B activity of the properdin system (7). More work is needed to fit the reaction described in this paper

into the entire C3-activator mechanism. The possibility exists that it constitutes an analogue to the fluid-phase reactions of the classical complement activation system (4). In this case, the three recognized factors of the bypass mechanism might enter into a multimolecular arrangement on the surface of the activating substance added to whole serum.

In view of the proposed effector function of HSFa, it seems probable that its activity is controlled by a regulatory principle in serum. A possible candidate is the C3b inactivator, which is known to degrade C3b enzymatically (8). Our results may aid clarification of the mechanism underlying congenital C3 hypercatabolism (9).

#### SUMMARY

The activity in human serum which is responsible for conversion of C3 proactivator (C3PA) to C3 activator was shown to reside in a 3S  $\alpha$ -globulin. The factor, called C3PA convertase (C3PAse), was obtained in partially purified form. For conversion of C3PA, C3PAse required participation of metal ions and of a C3 fragment, which in physicochemical and antigenic properties resembled C3b. Isolated, native C3 failed to substitute for the fragment, but did restore the impaired C3 activator system in hydrazine-treated serum. Unlike native C3, the C3 fragment initiated conversion of C3PA in whole serum. A hypothetical concept which envisions the C3 fragment as effector of C3PAse has been proposed.

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